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# Effects of salinity and temperature on reproductive and life span characteristics of clonal *Artemia*. (International Study on *Artemia*. LXVI)

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**Abstract**

An apomictic clone of the tetraploid parthenogenetic *Artemia* population from M. Embolon (Thessaloniki, Greece) was assayed for 10 reproductive and life span characteristics under laboratory conditions (in various salinity and temperature regimes). Salinity was proved to have significant impact on the majority of the characters used in this study. Discriminant function analysis gave an overall prediction of 97.32% over the three salinities (50, 80 and 120 ppt). The temperature of 30 °C seemed to be an extreme one affecting significantly nearly all of the studied variables. The overall prediction according to the discriminant analysis was 94.69% among the three temperatures (22, 26 and 30 °C). The clone performed best at 80 ppt and 22 °C. The data presented in this study may generate useful suggestions to investigate the potentiality of using a single genetic lineage in order to visualize the effects of different environmental cues on a specific clone.

## Introduction

The brine shrimp *Artemia* consists of a number of sexual species and a large number of obligately parthenogenetic strains inhabiting saline and hypersaline coastal or inland lakes. All bisexual species are diploid while asexual populations may be diploid, polyploid or mixtures of different ploidies. Diploid parthenogens are characterized by automixis, i.e., they are capable of limited meiotic recombination and therefore usually polyclonal. Polyploid asexual *Artemia* populations are apomictic (meiosis is totally suppressed) and therefore mainly monoclonal (for extensive reviews see Barigozzi, 1974; Browne & Bowen, 1991; Triantaphyllidis et al., 1998; Abatzopoulos et al., 2002).

Several studies have estimated environmental and genetic components of variance for life span and reproductive traits of *Artemia* (Browne et al., 1984; Wear & Haslett, 1986; Wear et al., 1986; Abatzopoulos et al., 1993; Triantaphyllidis et al., 1995; Browne & Wanigasekera, 2000; Browne et al., 2002). The majority of them have focused on temperature and salinity which are of the most important physical parameters affecting the life history of hypersaline organisms.

*Artemia* has been proven to be a model organism, offering substantial advantages for investigating the effects of temperature and salinity on life span and reproductive characters (Browne et al., 1984; Triantaphyllidis et al., 1995; Barata et al., 1996; Browne & Wanigasekera, 2000). Also, the combined effects of temperature and salinity on survival and reproduction of *Artemia* have been studied by several research teams mainly in laboratory conditions (Vanhaecke et al., 1984; Wear & Haslett, 1986; Wear et al., 1986; Vanhaecke & Sorgeloos, 1989; Triantaphyllidis et al., 1995; Browne & Wanigasekera, 2000).

Although monoclonal populations produced by apomictic parthenogenetic *Artemia* (i.e., lineages derived from a single mother) could be used successfully for studying phenotypic traits, this approach has been poorly exploited till now (see Browne et al., 2002).

In this study we tried to document the phenotypic expressions (over 10 life history traits) of a single genotypic lineage exposed to variable levels of two major environmental components: salinity and temperature. Discriminant analysis was used to define possible grouping based on the different salinity and temperature regimes.

## Material and methods

### *Clonal material*

An *Artemia* clone was isolated from the parthenogenetic population of M. Embolon saltworks (Thessaloniki, Greece – *Artemia* Reference Center – Ghent, Belgium – code No. 1420). This asexual population is tetraploid and apomictic (Abatzopoulos et al., 1986, 1987). The abbreviation $ME_1$ has been assigned to this clone.

### *Culture conditions*

Solutions of three different salinities, i.e., 50, 80 and 120 ppt were prepared using Instant Ocean® (Synthetic Sea Salts 1998, Aquarium Systems). The salinity was measured by a temperature-controlled ATAGO refractometer. The three experimental temperatures, i.e., 22, 26 and 30 °C (±0.5 °C) were maintained in thermostatically controlled water baths. Stock cultures of $ME_1$ (1 individual/4 ml) were kept in 1 l cylindroconical glass jars maintained at 22 °C. The salinity of the culture medium was 80 ppt. Animals were fed with 75% of yeast-based diet LANSY-PZ (INVE Aquaculture NV, Belgium) and 25% *Dunaliella tertiolecta* according to Triantaphyllidis et al. (1995). Approximately 50% of the culture medium was replaced by fresh medium every 7 days in the mass or stock cultures and every 4 days for the cultures in the 50-ml plastic cylindroconical tubes.

### *Experimental design*

#### *Salinity treatment*

The reproductive performance of $ME_1$ was tested at three selected salinities (i.e., 50, 80 and 120 ppt) at a constant temperature of 22±0.5 °C. Nauplii were transferred and acclimatized to the three different salinities (±1000 nauplii for each salinity) and kept in mass cultures. When the animals showed signs of ovarian development, they were removed from the mass culture and placed individually in 50-ml plastic cylindroconical tubes containing 40 ml of 0.45-$\mu$m filtered synthetic medium. Approximately 40 females were examined for each salinity. The tubes were examined every 2 days for offspring production or deaths. Reproductive and life span characteristics were determined according to Browne et al. (1984, 1988).

#### *Temperature treatment*

The reproductive performance of the $ME_1$ clone was tested at three selected temperatures (i.e., 22, 26 and 30 °C) at constant salinity of 80 ppt. Nauplii were transferred to the different temperatures (±1000 nauplii for each temperature) and maintained in mass cultures until they reached sexual maturity (ovarian development). Approximately 40 mature females were tested for each temperature, placed individually in 50-ml plastic cylindroconical tubes with 40 ml of 0.45-$\mu$m filtered synthetic medium of the appropriate temperature and the rest of the procedure was the same as described above for the salinity treatment.

The reproductive and life span characteristics scored for both treatments are the following: total number of offspring, number of broods, days between broods, offspring per reproductive day, offspring per brood, percentage of encysted embryos, pre-reproductive period, reproductive period, post-reproductive period and life span.

### *Statistical analyses*

Reproductive and life span characteristics were analyzed by standard single-factor ANOVA, where variances are assumed to be homogeneous (Sokal & Rohlf, 1981). Normality and homogeneity of group variances were checked by Kolmogorov–Smirnov and Bartlett's test. For some of the variables, numbers were log- or square root-transformed to satisfy assumptions of normality and homogeneity (Triantaphyllidis et al., 1995).

The 10 reproductive and life span variables determined in all individuals were used to establish relationships among the different treatments applied on the same *Artemia* clone through discriminant analysis. The rationale for using this approach is described in Triantaphyllidis et al. (1995) and Kachigan (1986).

*Table 1.* Mean (±S.D.) of various reproductive and life span characteristics for the parthenogenetic *Artemia* clone $ME_1$ reared at three different salinities (temperature was 22 °C). Significant differences were determined by ANOVA test ($P < 0.05$). Values in each line that share the same letter are not significantly different

| Characteristics | Salinity (ppt) | | |
|---|---|---|---|
| | 50 | 80 | 120 |
| Total number of offspring | $180.60^a$ | $482.03^b$ | $286.54^c$ |
| | (119.94) | (288.89) | (136.81) |
| Number of broods | $4.45^a$ | $4.70^a$ | $3.94^a$ |
| | (1.78) | (2.03) | (1.43) |
| Days between broods | $6.26^a$ | $4.30^b$ | $6.99^a$ |
| | (2.40) | (1.46) | (2.18) |
| Offspring per reproductive day | $6.56^a$ | $24.24^b$ | $11.82^c$ |
| | (5.01) | (9.42) | (9.02) |
| Offspring per brood | $36.54^a$ | $93.31^b$ | $71.57^c$ |
| | (16.84) | (28.65) | (26.35) |
| Percentage of encysted embryos | $14.61^a$ | $71.20^b$ | $87.39^c$ |
| | (5.10) | (10.26) | (8.86) |
| Pre-reproductive period* | $31.95^a$ | $33.14^b$ | $41.11^c$ |
| | (0.93) | (0.89) | (0.76) |
| Reproductive period* | $29.90^a$ | $21.81^b$ | $28.83^a$ |
| | (5.01) | (3.96) | (3.38) |
| Post-reproductive period* | $2.58^a$ | $1.51^b$ | $0.49^c$ |
| | (1.11) | (1.07) | (0.66) |
| Life span* | $64.43^a$ | $56.46^b$ | $70.43^a$ |
| | (5.28) | (4.07) | (7.28) |
| Number of females scored | 40 | 37 | 35 |

* In days.

Two analyses were carried out: in the first, the predefined groups were the salinities of 50, 80 and 120 ppt, and in the second, the different groups defined *a priori* at the temperatures of 22, 26 and 30 °C. These analyses have been performed using a standard procedure where all the selected variables were entered into the model simultaneously. The ranking of the variables according to their relative importance in discriminating different culture treatments was based upon *F*- and *P*-values.

Statistical analyses were performed with STATISTICA, release 5.1.

## Results

### *Salinity treatment*

Six reproductive and four life span characteristics from the parthenogenetic clone $ME_1$ cultured at three different salinities are summarized in Table 1. Statistical analysis using ANOVA indicated that significant differences exist among the different salinity treatments in most of the characters studied (see Table 1).

Comparisons of reproductive and life span variables in the three salinities revealed that six out of the 10 characters studied, were statistically different at 50, 80 and 120 ppt, i.e., the total number of offspring, offspring per reproductive day, offspring per brood, percentage of encysted embryos, pre- and post-reproductive period. Only one character, number of broods, was stable in the three salinities tested. Two variables, percentage of encysted embryos and pre-reproductive period, were significantly affected by salinity, exhibiting a parallel increase with salinity elevation. On the contrary, post-reproductive period decreased as salinity became higher. At 80 ppt, total offspring, offspring per reproductive day and offspring per brood showed the highest values (Table 1) indicating that the best reproductive performance of $ME_1$ clone was observed at this salinity (for the rationale of this statement see discussion). The reproductive period appeared rather stable although a significant decrease at 80 ppt was unexpected and difficult to perceive (see discussion). The same is valid for the life span of the clone at 80 ppt where the lowest values were observed.

Discriminant analysis, based on the three different culture salinities as a separator factor, revealed one discriminant function for each salinity and gave totally 97.32% prediction among the three salinities (Table 2). In particular, the prediction of the model in 120 ppt was 100%, while the prediction values in 50 and 80 ppt were 97.50 and 94.59%, respectively. All the reproductive and life span characteristics were used to construct the discrimination model, with the exception of life span; this variable was highly correlated with the reproductive period and was removed, as redundant. The variables contributing most to the discrimination model (according to *F*- and *P*-values) were total number of offspring, offspring per reproductive day and offspring per brood.

Discriminant analysis results for every individual produced a 2-D scatterplot (Fig. 1). It is obvious that three distinct clusters were formed; one for each culture salinity. The group consisting of individuals raised in 120 ppt was placed far from the other two groups. This fact implies that the output of individuals raised in 50 and 80 ppt was closer than the respective of the individuals cultured in 120 ppt.

*Table 2.* Discriminant analysis of the reproductive and life span characteristics determined in parthenogenetic *Artemia* clone $ME_1$ reared at different salinities. Unstandardised coefficients from the discriminant functions and predicted classifications are presented: 100% prediction means that with the variables used, 100% discrimination of the three salinities can be obtained. A – total number of offspring; B – number of broods; C – days between broods; D – offspring per reproductive day; E – offspring per brood; F – percentage of encysted embryos; G – pre-reproductive period; H – reproductive period; I – post-reproductive period

| Variables | Discriminant function coefficients for the various salinities | | |
|---|---|---|---|
| | 50ppt | 80ppt | 120ppt |
| A | 0.02472 | 0.04579 | 0.05143 |
| B | 7.85939 | 7.22740 | 1.55210 |
| C | 13.12100 | 14.70047 | 14.28341 |
| D | 2.96154 | 3.51115 | 3.78354 |
| E | −0.88584 | −1.00078 | −1.17422 |
| F | 34.66897 | 52.81785 | 70.90108 |
| G | 45.66936 | 47.90837 | 59.70777 |
| H | −1.56595 | −1.88112 | −1.15255 |
| I | 1.14927 | −0.43004 | −2.43598 |
| Constant | −765.53400 | −848.32000 | −1283.10000 |
| Predicted classifications | | | |
| 50 ppt | 97.50% | | |
| 80 ppt | 94.59% | | |
| 120 ppt | 100.00% | | |
| Total | 97.32% | | |

*Temperature treatment*

Ten reproductive and life span characteristics from the clonal *Artemia* $ME_1$ cultured at three different temperatures (22, 26 and 30 °C) and their statistical differences are presented in Table 3.

ANOVA revealed that the three different temperatures affected significantly the performance of the clone, according to reproductive and life span characteristics ($P < 0.05$). Individuals raised at 22 °C showed the best output for the majority of reproductive and life span characteristics; specifically, the total number of offspring, offspring per brood and reproductive period which contributed most were statistically different in the three different culture temperatures (Table 3). Three other variables (percentage of encysted embryos, pre-reproductive period and post-reproductive period) were not statistically different in individuals raised at 22 and 26 °C, while their values were statistically different compared with those obtained in 30 °C. Offspring per reproductive day was the only variable which presented no statistical differences in the three groups. According to the reproductive and life span results of this study, individuals cultured at 22 °C were closer to those cultured at 26 °C, than those at 30 °C.

Discriminant analysis, based on the three different culture temperatures as a separator factor, revealed one discriminant function for each temperature and gave totally 94.69% prediction among the three temperatures (Table 4). It should be mentioned that the prediction of the model in 26 and 30 °C was 100%, while it dropped to 82.36% at 22 °C. All the reproductive and life span characteristics were used to construct the discrimination model, with the exception of life span; this variable was highly correlated with the reproductive period and was removed as redundant. The reproductive and life span variables, which contributed mostly to the discrimination model (based on *F*- and *P*-values), were: the total number of offspring, offspring per brood and reproductive period.

Discriminant analysis results for every individual produced a 2-D scatterplot (Fig. 2). It is obvious that three clusters were formed; one for each culture temperature. The group consisting of individuals raised in 30 °C was placed far from the other two groups, which overlapped. This implies that the output of individuals raised in 22 and 26 °C was closer than the respective of the individuals cultured at 30 °C. In addition, 26 and

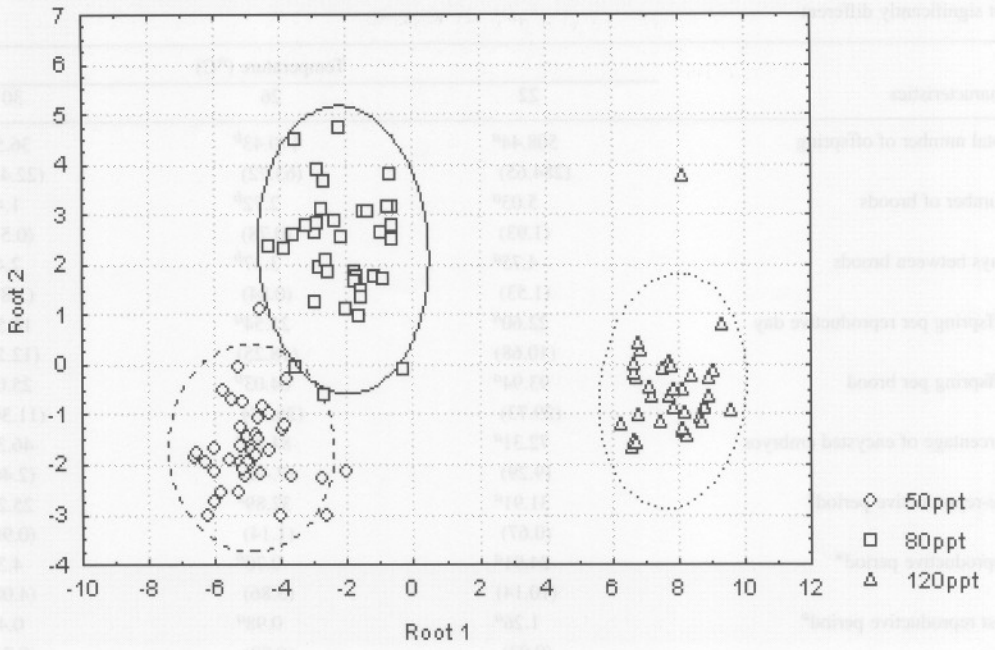


*Figure 1.* Scatterplot resulting from the discriminant analyses (canonical scores) on parthenogenetic females ($ME_1$ clone) when using salinity as separator factor. Border lines represent 95% confidence level.

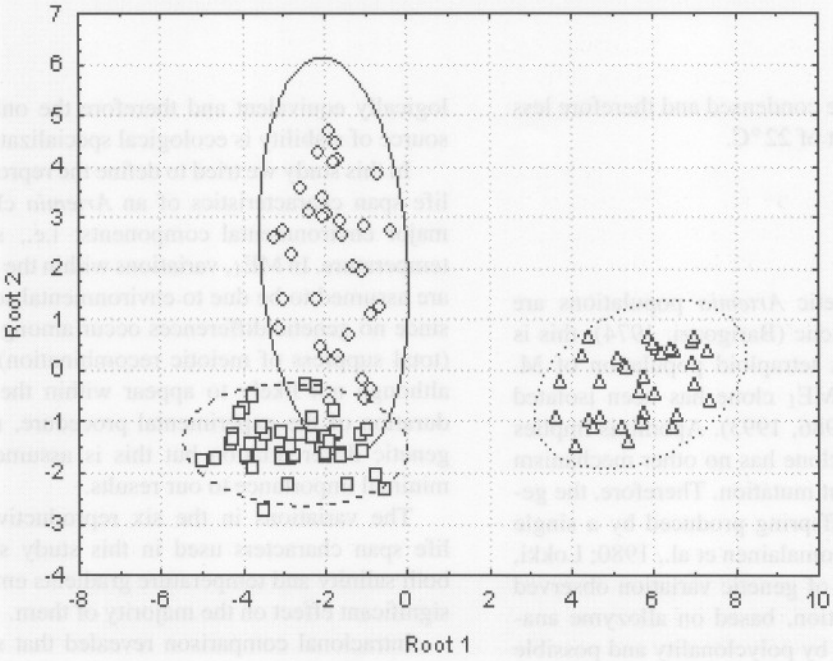


*Figure 2.* Scatterplot resulting from the discriminant analyses (canonical scores) on parthenogenetic females ($ME_1$ clone) when using temperature as separator factor. Border lines represent 95% confidence level.

*Table 3.* Mean (±S.D.) of various reproductive and life span characteristics for the parthenogenetic *Artemia* clone $ME_1$ reared at three different temperatures (salinity was 80 ppt). Significant differences were determined by ANOVA test ($P < 0.05$). Values in each line that share the same letter are not significantly different

| Characteristics | Temperature (°C) | | |
|---|---|---|---|
| | 22 | 26 | 30 |
| Total number of offspring | 508.44$^a$ | 170.43$^b$ | 36.52$^c$ |
| | (284.65) | (63.72) | (22.42) |
| Number of broods | 5.03$^a$ | 2.72$^b$ | 1.45$^c$ |
| | (1.93) | (0.78) | (0.51) |
| Days between broods | 4.75$^a$ | 3.37$^b$ | 2.43$^c$ |
| | (1.53) | (0.84) | (1.81) |
| Offspring per reproductive day | 22.60$^a$ | 21.54$^a$ | 16.59$^a$ |
| | (10.68) | (18.25) | (12.21) |
| Offspring per brood | 93.94$^a$ | 64.03$^b$ | 25.06$^c$ |
| | (29.73) | (26.34) | (11.30) |
| Percentage of encysted embryos | 72.31$^a$ | 84.41$^a$ | 46.33$^b$ |
| | (9.29) | (7.48) | (2.40) |
| Pre-reproductive period* | 31.91$^a$ | 32.89$^a$ | 25.27$^b$ |
| | (0.67) | (1.14) | (0.98) |
| Reproductive period* | 24.91$^a$ | 9.70$^b$ | 4.33$^c$ |
| | (10.14) | (3.86) | (4.08) |
| Post reproductive period* | 1.26$^a$ | 0.98$^a$ | 0.48$^b$ |
| | (0.93) | (0.88) | (0.71) |
| Total life span* | 58.09$^a$ | 43.57$^b$ | 30.09$^c$ |
| | (10.40) | (4.12) | (4.23) |
| Number of females scored | 34 | 47 | 33 |

* In days.

30 °C groups were more condensed and therefore less variant compared to that of 22 °C.

## Discussion

Polyploid parthenogenetic *Artemia* populations are considered to be apomictic (Barigozzi, 1974); this is the case for the Greek tetraploid population of M. Embolon from which $ME_1$ clone has been isolated (Abatzopoulos et al., 1986, 1993). Apomixis implies that a parthenogenetic clone has no other mechanism for genotypic change but mutation. Therefore, the genetic make-up of all offspring produced by a single female are identical (Suomalainen et al., 1980; Lokki, 1983). The lower level of genetic variation observed in M. Embolon population, based on allozyme analysis, may be explained by polyclonality and possible selective differences among clones (Abatzopoulos et al., 1993). According to Young (1983) there is considerable evidence that coexisting clones may not be ecologically equivalent and therefore the only plausible source of stability is ecological specialization.

In this study we tried to define the reproductive and life span characteristics of an *Artemia* clone to two major environmental components: i.e., salinity and temperature. In $ME_1$, variations within the populations are assumed to be due to environmental sources only, since no genetic differences occur among individuals (total suppress of meiotic recombination). Mutation, although not likely to appear within the very short duration of the experimental procedure, may induce genetic differentiation but this is assumed to be of minimal importance to our results.

The variations in the six reproductive and four life span characters used in this study showed that both salinity and temperature gradients employed had significant effect on the majority of them.

Intraclonal comparison revealed that salinity had a major impact on five out of six reproductive traits studied (only the number of broods seemed to be unaffected). In the salinity of 80 ppt the clone exhibited

*Table 4.* Discriminant analysis of the reproductive and life span characteristics determined in parthenogenetic *Artemia* clone $ME_1$ reared at different temperatures. Unstandardised coefficients from the discriminant functions and predicted classifications are presented: 100% prediction means that with the variables used, 100% discrimination of the three salinities can be obtained. A – total number of offspring; B – number of broods; C – days between broods; D – offspring per reproductive day; E – offspring per brood; F – percentage of encysted embryos; G – pre-reproductive period; H – reproductive period; I – post-reproductive period

| Variables | Discriminant function coefficients for the various temperatures | | |
|---|---|---|---|
| | 22 °C | 26 °C | 30 °C |
| A | −0.05266 | −0.06458 | −0.01735 |
| B | 24.67367 | 30.11595 | 22.43797 |
| C | 12.53845 | 14.51999 | 13.70276 |
| D | 0.66419 | 0.64192 | 0.65624 |
| E | −0.11107 | −0.11470 | −0.29082 |
| F | 10.85867 | 16.15654 | 12.73700 |
| G | 35.56271 | 37.12116 | 28.69688 |
| H | −3.99895 | −5.51038 | −4.76378 |
| I | −5.96708 | −6.77265 | −5.30065 |
| Constant | −599.73000 | −651.30000 | −389.66400 |
| Predicted classifications | | | |
| 22 °C | 82.36% | | |
| 26 °C | 100.00% | | |
| 30 °C | 100.00% | | |
| Total | 94.69% | | |

the highest reproductive output expressed as total offspring per female (Table 1). This is most obvious when considering the offspring per reproductive day and offspring per brood, variables that were significantly higher at 80 ppt ($P < 0.05$) while broods were more frequent (Table 1). In this sense, we consider that this specific clone performs very well at the salinity of 80 ppt. Considering that *Artemia* follows the r-strategy, the most successful clonal response would be the production of a great number of offspring within the shortest possible period. For similar results see Triantaphyllidis et al. (1995).

It is obvious that elevation of salinity induces oviparity, which is expressed as significant increase of the produced encysted embryos (Table 1, $P < 0.05$).

Life span characteristics are also affected by salinity. Higher salinities caused delay in development (e.g., in 120 ppt the first brood occurred after 41 days).

An interesting observation was that the $ME_1$ clone exhibited shorter life span when reared at 80 ppt. This event seemed to be inconsistent with the high reproductive performance of the clone in this salinity. However, the animals having an elevated reproductive output within a short period of time may utilize the available energy reserves, resulting in increased mortality. The discriminant analysis grouped quite distinctly the three salinity treatments with 120 ppt to differentiate mostly (see Table 2 and Fig. 1). This supports further the significantly different results as they were computed by ANOVA.

One may ask how the clone would respond in salinities higher than 120 ppt. In fact, salinities of 160 and 200 ppt were investigated but there were either major crashes of the cultures or the pre-reproductive period was enormously prolonged so that the animals did not reach sexual maturity. Similar results have been recorded by other workers for parthenogenetic *Artemia* (Triantaphyllidis et al., 1995; Browne & Wanigasekera, 2000).

The present study shows that temperature has a major influence on five out of the six reproductive traits studied; only the offspring per reproductive day seemed to be stable in all three temperature treatments (Table 3). At 22 °C the clone exhibited the highest reproductive output expressed as total number of offspring per female. This was, also, supported by the largest number of broods and especially by the offspring per brood; both variables are significantly

higher at 22 °C ($P < 0.05$). Pooling these results with those from salinity treatments it is obvious that the $ME_1$ displays its 'best performance' at 22 °C in 80 ppt; for this reason this combination of temperature and salinity was selected as 'control' in the two batteries of experiments.

An interesting point is that the percentage of encysted embryos appeared to be significantly lower in 30 °C, although, one would expect the reverse since this temperature is stressful for this clone. A simple explanation is the fact that the number of broods at 30 °C is extremely low and it is well known that *Artemia* females in their first and/or second brood are mainly ovoviviparous (Lenz, 1987, and references therein).

Life span characteristics were also affected by temperature. All characters were significantly different ($P < 0.05$) at 30 °C (Table 3). Life span, reproductive period and post reproductive period decreased significantly. These results were expected since 30 °C is an extreme temperature in the thermal history of this *Artemia* population, i.e. *Artemia* in M. Embolon saltworks is never subjected to the temperature of 30 °C for such long periods of 30 days as applied in these laboratory cultures. Contrary to the three previous life span characteristics, maturation was accelerated (expressed as shorter pre-reproductive period, see Table 3). Discriminant analysis grouped quite distinctly the temperatures of 26 and 30 °C while for 22 °C the predicted classification is weaker (82.36%, Table 4 and Fig. 2).

Unfortunately, relevant studies to this one that could be used for comparison purposes are extremely scarce. Apomictic *Artemia* clones are strongly suggested to be used in future studies for scoring *Artemia* response to different environmental components.

The data, derived from the study of reproductive and life span characteristics of the $ME_1$ *Artemia* clone cultured in different salinities and temperatures, provide evidence that this parthenogenetic clone exhibits its best performance at 80 ppt and 22 °C. The M. Embolon parthenogenetic population is multiclonal and, therefore, other clones may perform better at temperatures around 30 °C; further studies are required in this direction.

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